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Differential Display RT PCR of Total RNA From Human Foreskin Fibroblasts for Investigation of Androgen-Dependent Gene Expression

E.M. Nitsche, A. Moquin, P.S. Adams, R.S. Guenette, J.N. Lakins, G.H.G. Sinnecker, K. Kruse, and M.P. Tenniswood

W.A. Jones Cell Science Center, Lake Placid, New York (E.M.N., A.M., P.S.A., R.S.G., J.N.L., M.P.T.); Department of Pediatrics, University of Luebeck, Germany (E.M.N., G.H.G.S., K.K.)

Male sexual differentiation is a process that involves androgen action via the androgen receptor. Defects in the androgen receptor, many resulting from point mutations in the androgen receptor gene, lead to varying degrees of impaired masculinization in chromosomally male individuals. To date no specific androgen regulated morphogens involved in this process have been identified and no marker genes are known that would help to predict further virilization in infants with partial androgen insensitivity.

In the present study we first show data on androgen regulated gene expression investigated by differential display reverse transcription PCR (dd RT PCR) on total RNA from human neonatal genital skin fibroblasts cultured in the presence or absence of 100 nM testosterone. Using three different primer combinations, 54 cDNAs appeared to be regulated by androgens. Most of these sequences show the characteristics of expressed mRNAs but showed no homology to sequences in the database. However 15 clones with significant homology to previously cloned sequences were identified. Seven cDNAs appear to be induced by androgen withdrawal. Of these, five are similar to ETS (expression tagged sequences) from unknown genes; the other two show significant homology to the cDNAs of ubiquitin and human guanylate binding protein 2 (GBP-2). In addition, we have identified 8 cDNA clones which show homologies to other sequences in the database and appear to be upregulated in the presence of testosterone. Four of these clones again are similar to ETS from unknown genes. Three differential expressed sequences that appear to be upregulated in the presence of testosterone show significant homology to the cDNAs of L-plastin and one to the cDNA of testican. This latter gene codes for a proteoglycan involved in cell social behavior and therefore of special interest in this context. The results of this study are of interest in further investigation of normal and disturbed androgen-dependent gene expression. © 1996 Wiley-Liss, Inc.

KEY WORDS: testicular feminization, testican, L-plastin, ubiquitin

INTRODUCTION

Male sexual differentiation is a complex process involving the interaction of multiple factors leading to a normal male phenotype in response to an XY genotype. One of these factors is the synthesis and action of androgens. Testosterone produced in the embryonic testis supports the differentiation of the Wolffian ducts into vas deferens, epididymis, and seminal vesicles [Siiteri et al., 1974]. After reduction by 5-α-reductase, 5-α-dihydrotestosterone (5-α-DHT) plays a major role in the development of the prostate, proximal urethra, and the virilization of the external genitalia [Siiteri et al., 1974; George et al., 1989; Peterson et al., 1979; Clark et al., 1990]. Like other steroid hormones [Härd et al., 1990], androgens act by binding to specific intracellular receptors, and the formation of the hormone receptor complex leads to a change in conformation of the receptor molecule with dimerization and exposure of the DNA binding domain. Thus, the androgen receptor complex can bind to androgen response elements and modulate the expression of androgen-sensitive genes [Liao et al., 1989; Trapman et al., 1988].

Mutations of the androgen receptor can lead to a variety of phenotypes with incomplete masculinization depending on the site and type of mutation. The clinical appearance of a patient with mutation in the androgen receptor gene ranges from complete testicular

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Address reprint requests to Esther M. Nitsche, Department of Pediatrics, University of Luebeck, Germany.

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feminisation with normal female phenotype through varying degrees of ambiguous genitalia to the patient with normal male phenotype presenting with infertility [Aiman et al., 1979, 1982; Kaufman et al., 1990; Grino et al., 1988; Madden et al., 1975; Kaufman et al., 1976; Pinsky et al., 1981]. However, infertility does not seem to be an obligate symptom in partial androgen insensitivity [Grino et al., 1988; Pinsky et al., 1981] and in families with a syndrome of partial androgen insensitivity and intact fertility there seems to be variability of the expression of the defect [Wilson et al., 1974].

A large number of qualitative and quantitative androgen receptor defects have been characterized by ligand binding assays [Brown et al., 1982; Kaufman et al., 1981, 1990, 1986, 1984, 1982, 1983; Pinsky et al., 1992; Quigley et al., 1995]. Following the cloning and sequencing of the androgen receptor cDNA [Trapman et al., 1988; Chang et al., 1988; Lubahn et al., 1988; Ris-Stalpers et al., 1994], many of these defects have been attributed to point mutations in the androgen receptor gene. However, the cellular effects of androgen receptor defects remain unclear. The predictability of the clinical consequences of particular mutations based on the knowledge of the site and type of the mutation is still not reliable. Since the decisions regarding the future gender role of the child and for surgical corrections must be made early in the patient's life, patterns of expressed marker-genes for the various partial functions of the androgen-receptor complex would be very useful. Therefore, the aim of the present study was to identify androgen-regulated genes involved in male sexual differentiation. This was done by differential display RT PCR [Liang et al., 1992, 1993; Bauer et al., 1993; Liang and Pardee, 1992] comparing the mRNA populations of human neonatal genital foreskin fibroblasts grown under serum-free conditions in presence or absence of 100 nM testosterone as a model. Human genital skin fibroblasts are known to express and respond to androgenic stimulation through the androgen receptor [Brown and Migeon, 1981].

MATERIALS AND METHODS

Cell Culture

Neonatal genital skin fibroblasts were obtained from foreskins after routine circumcision and informed consent of the parents. The sample was stored in sterile phosphate buffered saline pH 7.4 at 4°C up to 48 h prior to culture. After mincing the tissue was digested with collagenase 1 h at 37°C (Collagenase D, final concentration approximately 0.85 Wunsch U/ml; Boehringer Mannheim, Indianapolis, IN). The primary fibroblasts were cultured in DMEM with high glucose and without phenol red (DMEM 13000 GIBCO BRL, Life Technologies, Inc., Grand Island, NY) with 25 mM HEPES,1 mM pyruvate, 40 mM $NaHCO_3$, 100 nM testosterone, and 10% fetal calf serum and passaged twice.

The third passage was performed at a density of 1×10^6 cells per cm^2 in 125 cm^2 tissue culture flasks into serum-free, phenol red-free media as previously described [Colletta et al., 1990] with minor modifications (DMEM 13000 GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 25 mM HEPES, 1 mM pyruvate, 40 mM $NaHCO_3$, 2 mg/l human fibronectin (Upstate Biotechnology, Inc., Lake Placid, NY), 10 μg/l EGF (Upstate Biotechnology, Inc., Lake Placid, NY), 5 mg/l human insulin (Sigma Chemical Company, St. Louis, MO), 5 mg/l human transferrin (Upstate Biotechnology, Inc., Lake Placid, NY), 1 nM copper sulfate, and 0.5 nM sodium selenite. Testosterone was added in 20 μl 90% ethanol (+T conditions) to a final concentration of 100 nM; control cells received 20 μl 90% ethanol alone (−T conditions). A second dose of testosterone was added after 24 h. After 48 h under differential conditions, the cells were harvested for RNA extraction. At this point they had reached approximately 60% confluency.

RNA Extraction

Total RNA was extracted using the lithium chloride urea procedure as previously described [Auffray and Rougeon, 1979] with minor modifications or using RNAzol B (Biotecx Laboratories, Houston, TX). The total RNA was treated with DNAse (Promega Corp., Madison, WI) as described by Liang et al. [1993].

RT PCR for mRNA of the Human Androgen Receptor (hAR) and Southern Blot

The primers for RT PCR (Table I) were chosen so that the PCR product would span introns of the androgen receptor gene preventing amplification of genomic DNA.

One microgram of total RNA was reverse-transcribed in a total volume of 20 μl containing 0.5 mM dNTPs, 10 mM DTT, 200 U reverse transcriptase (Superscript II GIBCO BRL), 0.5 μM huAR downstream primer (Table I) in 50 mM TrisHCl, 40 mM KCl, and 6 mM $MgCl_2$ (pH 8.3) for 1 h at 42°C followed by an incubation of 30 min at 50°C. Five microliters of the RT product were used for PCR amplification, which was carried out in a total volume of 50 μl containing 1.2 mM dNTPs, 1 μM each of huAR downstream and upstream primer (Table I) in 10 mM TrisHCl, 50 mM KCl, and 7.0 mM $MgCl_2$ (pH 8.4). The cycling conditions, following a hot start, were the incubation continued for 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C followed by 15 min at 72°C, and cooling to 4°C for at least 5 min. Electrophoretic separation was performed on a 1% agarose gel in 40 mM Tris-acetate, 1 mM EDTA (pH 7.7) running buffer. The DNA was transferred to a nylon membrane (Genescreen plus, Du Pont NEN Research Products, Boston, MA) overnight using 3 M NaCl, 0.3 M Na_3Citrate (pH 7.0) transfer buffer and hybridized with a huAR internal probe (Table I), end-labeled with γ^{32}P-dATP (3000 Ci/mmol, Du Pont NEN Research Products, Boston, MA). The membrane was exposed to X-ray film for 24–48 h. Total RNA from the androgen receptor positive human prostate carcinoma cell line LNCaP served as positive control; total RNA from the androgen receptor negative cell line PC3 served as a negative control.

TABLE I. Primers and Internal Probe Used in RT PCR and Southern Blot of the Human Androgen Receptor (huAR) mRNA

hAR downstream	5′-ACTACACCTGGCTCAATGGC-3′
hAR upstream	5′-TGGATGGATAGCTACTCCGG-3′
hAR internal	5′-CTCTCACATGTGGAAGCTGC-3′

DD RT PCR

Differential display RT PCR was performed using the RNAmap kit by GenHunter Corp. (Brookline, MA); 2 μg of total RNA were reverse-transcribed in a total volume of 20 μl using 20 μM dNTPs, 1 μM downstream primer (Table II) in 50 mM TrisHCl, 75 mM KCl, 3 mM $MgCl_2$, and 5 mM DTT (pH 8.3), using 200 U reverse transcriptase (Superscript II, GIBCO BRL) for 1 h at 37°C. The reaction was stopped by heating to 95°C for 5 min at the end of the incubation. Two microliters of the RT mixture per condition (+T, −T) were PCR amplified in a total volume of 20 μl containing either 1 μM downstream primer and 0.2 μM upstream primer (Table II) or 0.4 μM upstream primer only, 2 μM dNTPs, 10 μCi ^{35}S-dATP dATP (1,300 Ci/mmol, Du Pont NEN Research Products, Boston, MA), and 2.5 U Taq polymerase (Amplitaq Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ) in 10 mM TrisHCl, 50 mM KCl, and 1.5 mM $MgCl_2$ (pH 8.4). Cycling conditions were 30 sec at 94°C, 2 min at 40°C, and 30 sec at 72°C for 40 cycles, followed by cooling to 4°C for at least 5 min. The PCR products were resolved by electrophoresis on a standard denaturing 6% acrylamide (VWR, Piscataway, NJ)/8 M urea (Sigma Chemical Comp., St. Louis, MO) gel with 0.03 M Tris-Borate, 0.72 μM EDTA (pH 8.3) running buffer at 1,500 V, 30 mA, and 40 W. Sequamark (Research Genetics, Huntsville, AL) was used as a molecular weight marker. The gels were dried on filter paper and exposed to X-ray film for 24 to 72 h. Bands of interest were cut out through the film and eluted by soaking in ddH_2O followed by heating to 100°C for 15 min, were ethanol precipitated, and were redissolved in 10 μl ddH_2O.

Five microliters of the eluted DNA were reamplified again using either 1 μM downstream primer and 0.2 μM upstream primer (Table II) or 0.4 μM upstream primer only in a total volume of 40 μl, using the same conditions as described above; 3 μl of the PCR product were electrophoresed on a 1% agarose gel in 40 mM Tris-acetate 1 mM EDTA (pH 7.7) running buffer. The PCR products were visualized by staining with ethidium bromide and UV transillumination and were reamplified if necessary.

Cloning of the PCR Products

The reamplified DNA was cloned using the Invitrogen TA cloning kit (Invitrogen, San Diego, CA). Clones were grown up in liquid cultures of LB broth containing 50 μg kanamycin/ml. The plasmids were isolated using the Wizard Miniprep kit by Promega Corp. (Madison, Wi). The size of the inserts was confirmed by restriction digestion using either EcoRI or NsiI followed by electrophoretic separation of the samples on a 1% agarose gel as described above.

TABLE II. Primers Used in Differential Display RT PCR

Downstream primer	T_{12}-MG
Upstream primer AP-1	5′-AGCCAGCGAA-3′
Upstream primer AP-2	5′-GACCGCTTGT-3′
Upstream primer AP-3	5′-AGGTGACCGT-3′

TABLE III. Primers Used for Sequencing of the Cloned Products

M 13 forward	5′-GACCGGCAGCAAAATG-3′
M 13 reverse	5′-GTACCAGTATCGACAA-3′

Sequencing of the cDNA Clones

Successfully cloned cDNAs were sequenced using the ABI Prism ready reaction Dyedeoxy Terminator Sequencing kit (Applied Biosystems, Inc., Foster City, CA) and M13 forward and reverse primer (Table III) in an ABI 370A automated sequencer.

RESULTS

The primary fibroblast cultures grew well under the described conditions. After changing to serum-free conditions, the division rate dropped. However, the cell morphology did not change significantly under serum-free conditions or after androgen withdrawal. In particular, signs of apoptosis were not observed after removal of testosterone from the culture medium. Observation of the cells up to 120 h under differential conditions showed that the cells kept dividing slowly and the morphology did not change during that period of time. The androgen receptor mRNA as measured by RT PCR was still present in the fibroblasts grown in the absence of testosterone for 48 h (Fig. 1).

Differential display with three different primer sets (Table II) showed an increase of several bands and the appearance of new bands in both the +T and −T conditions (Fig. 2). Interestingly, adding the upstream primer only to the PCR reaction showed bands in addition to those seen where both primers were added. Despite DNAse treatment, in every experiment some bands were amplified even in the absence of reverse transcriptase. Corresponding bands in the lanes of the same RNA amplified following reverse transcription were ignored to avoid false positive results due to priming of genomic DNA. A total of 54 bands appeared to be significantly different between the two conditions and were isolated from the gels. Nineteen bands were taken from the lanes from dd RT PCR from total RNA from cells grown in the presence of testosterone and 35 bands from the testosterone-depleted conditions (Table IV).

TABLE IV. Bands Eluted From Acrylamide Gels

Downstream primer	New bands/and bands increased with testosterone	New bands/and bands increased without testosterone
T_{12}-MG, AP-1	3	20
AP-1 only	7	2
T_{12}-MG, AP-2	3	2
AP-2 only	3	—
T_{12}-MG, AP-3	—	5
AP-3 only	—	—
T_{12}-MG	3	6
	19	35

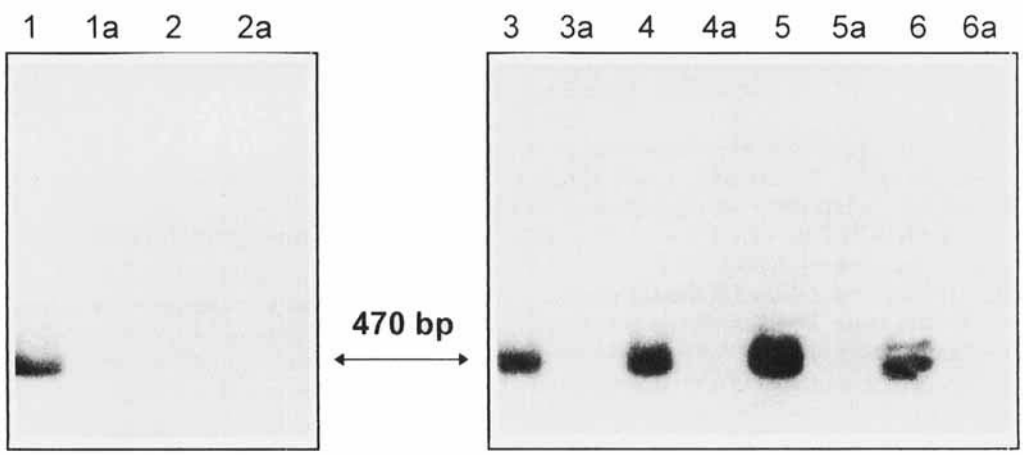

Fig. 1. Southern blot following RT PCR for androgen receptor mRNA. 1, positive control (total RNA from LNCap cells); 1a, positive control, no reverse transcriptase added; 2, negative control (total RNA from PC3 cells); 2a, negative control, no reverse transcriptase added; 3 + 4, total RNA from genital skin fibroblasts grown with testosteron; 3a + 4a, total RNA as lane 3 + 4, no reverse transcriptase added; 5 + 6, total RNA from genital skin fibroblasts grown without testosteron; 5a + 6a, total RNA as lane 5 + 6, no reverse transcriptase added.

After reamplification, cloning, and sequencing, most cDNAs showed no homology to known sequences but many showed the characteristics of expressed mRNAs. These include the presence of both upstream and downstream primer sequences and the poly(A) addition motif AATAAA in the amplified cDNA in close proximity (15–30 base pairs) to the poly(A) sequence. These sequences are worthy of future characterization by RNAse protection assay (RPA) or northern blot analysis.

However, 15 sequences were highly homologous to previously sequenced genes; 8 of these appeared to be upregulated by testosterone, while 7 appeared to be induced after testosterone withdrawal (Table V). From the bands that are upregulated in the presence of testosterone, we have cloned four sequences that show significant homology to previously reported expression tagged sequences (ETS) which to date have no known function. In addition, one sequence shows near identity to exon 4 of human alcohol dehydrogenase 5. Three cDNAs show homology to the same gene and were significantly homologous to the L-Plastin cDNA bp 158–539, while one sequence shows significant homology to the Testican cDNA bp 1970–2113 (Table V).

From the bands that appeared to be upregulated after removal of testosterone deprivation we have cloned five partial cDNAs. One of the sequences shows equal homology to two different ETSs (Table V). We have also identified sequences similar to known genes including

TABLE V. Cloned Sequences With Significant Homologies to Previously Cloned cDNAs That Are Entered in Databases*

Clone number +T	Clone number −T	Size of the clone bp	Amount of homology bp/bp	Homology
39.17		400	371/400	L-Plastin mRNA, bp 158–538
40.4		394	278/394	L-Plastin mRNA, bp 158–439
41.10		397	154/164	L-Plastin mRNA, bp 377–539
47.10		336	140/146	part. cDNA clone emb Z 41211, bp 36–156
49.6		169	101/112	hu cDNA gb R02550, bp 3–114, or
			99/121	hu ADH 5 Exon 4, bp 195–314
51.2		280	138/144	hu keratinocyte cDNA clone 647, bp 247–356
59.4		240	146/172	hu cDNA clone emb Z33551, bp 61–232
59.5		245	138/144	Testican mRNA, bp 1970–2113
	27.2	176	101/102	part. cDNA clone emb Z395779, bp 45–183
		225	101/102	part. cDNA clone emb F01802, bp 45–183
	32.2	362	243/250	Ubiquitin mRNA, bp 2012–2261
	32.6	351	252/270	hu GBP-2 isoform 1 mRNA, bp 62–331
	45.1	330	317/323	ORF mRNA, gb D31766, bp 1200–1522
	57.7	296	99/118	hu cDNA clone emb Z36759, bp 123–240
	61.2	392	191/221	hu cDNA clone emb Z19394, bp 92–312
	61.10	267	158/163	hu cDNA clone gb T32602, bp 1–163

* For the expression tagged sequences so far of unknown function, the database (emb, EMBL DNA sequence database; gb, genBank DNA sequence database) is given.

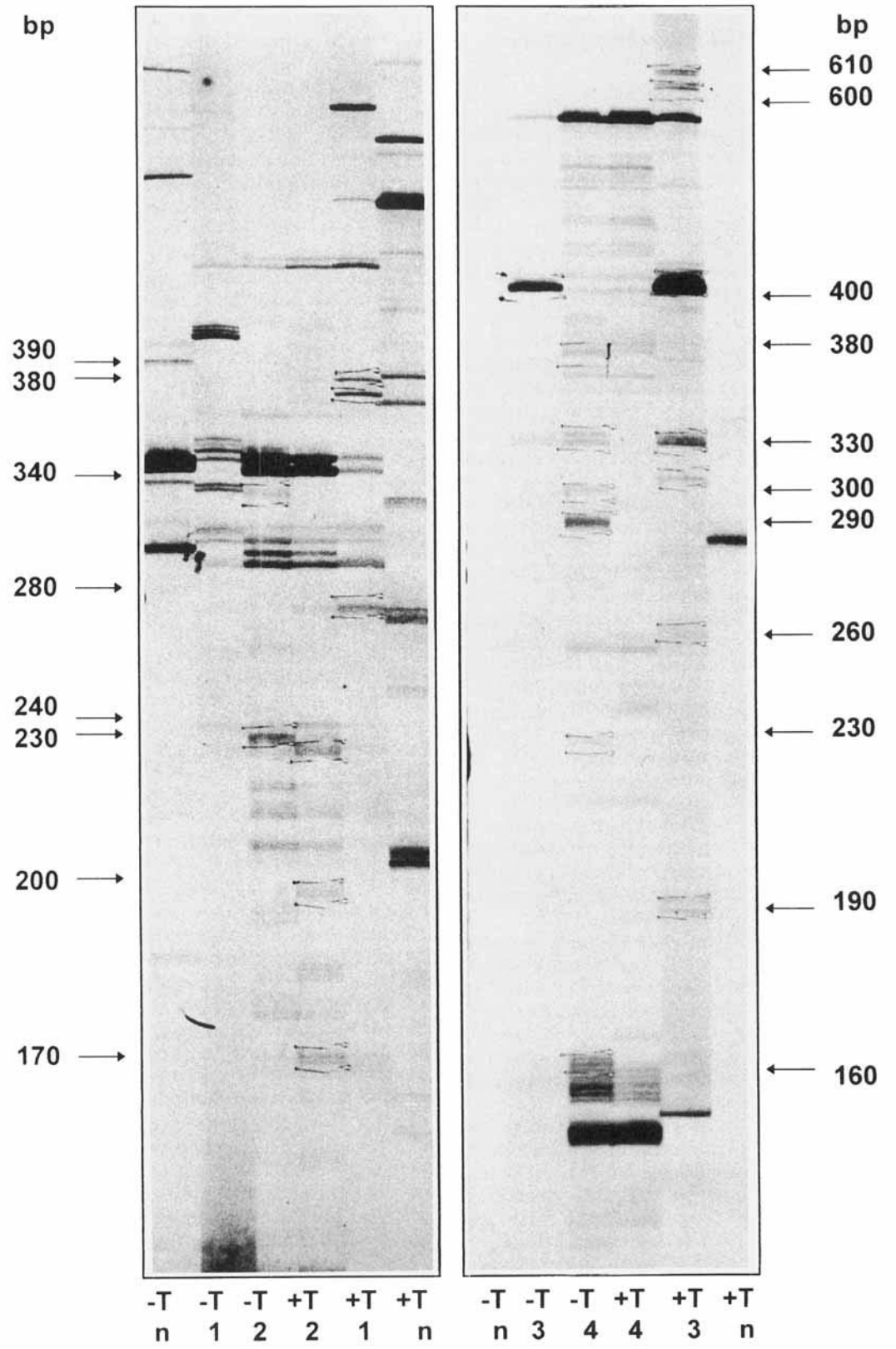

Fig. 2. Autoradiography of dd RT PCR acrylamide gel. Exposure time 24 h. +T, total RNA from cells grown in the presence of 100 nM testosterone. −T, total RNA from cells grown in the absence of 100 nM testosterone; n, no reverse transcription; 1, PCR with primer AP-1 only; 2, PCR with primers T_{12}MG and AP-1; 3, PCR with primer AP-2 only; 4, PCR with primers T_{12}MG and AP-2.

cDNAs homologous to ubiquitin cDNA bp 2012–2261 and human guanylate binding protein 2 isoform 1 (GBP-2 isoform 1) bp 62–331.

DISCUSSION

In the present study we have established that neonatal genital skin fibroblasts cultured in serum-free medium, even in the absence of testosterone, maintain their morphology but decrease their division rate. These cells have previously been shown to express the androgen receptor [Tilley et al., 1990] and have been used to study defects of the receptor protein [Pinsky et al., 1981; Schweikert et al., 1987, 1989].

In many tissues and cell lines the androgen receptor itself has been shown to be androgen-dependent and appears to be upregulated by androgens at the protein level due to reduced degradation even though the mRNA level may decrease in the presence of androgens [Shan et al., 1990; Krongrad et al., 1991]. Using the non-metabolizable androgen mibolerone, the maximum effect on mRNA expression in LNCaP cells was found 48 h after addition of androgen resulting in a drop in mRNA levels to 30% of the initial levels; other cells showed a similar behavior [Wolf et al., 1993]. However, this does not appear to be true for normal foreskin fibroblasts. An additional cell type-specific protein involved either in the lack of suppression of androgen receptor expression in fibroblasts or the repression in other cell types has been postulated [Wolf et al., 1993]. The results of RT PCR for huAR mRNA and subsequent southern blots demonstrate, that foreskin fibroblasts in culture also continue to express androgen receptor mRNA in serum-free medium in the presence or absence of 100 nM testosterone during the study period of 48 h and up to 96 h. Investigations in the Tfm mouse have suggested that genes regulated by the mesenchymal androgen receptor play a major role in the differentiation of the male urogenital tract, while the epithelial androgen receptor is neither necessary nor sufficient for male sexual differentiation [Cunha et al., 1987].

Using three primer combinations we have isolated several that appeared to be differentially expressed in human neonatal foreskin fibroblasts in the presence or absence of testosterone. Most of these sequences have the characteristics of expressed mRNAs, but do not show any homology to known genes. In addition to this we have identified a few candidate genes which may be specifically regulated by androgens.

Interestingly almost twice as many bands were observed to be differentially expressed in the mRNA of neonatal foreskin fibroblasts cultured for 48 h in the absence of androgens. This indicates that androgens may repress the expression of specific genes during this stage of sexual development in the male neonate. At this time (often referred to as mini-puberty), the serum testosterone levels reach almost pubertal values. The combination of androgen repression of one or more specific genes involved in the regulation of the development of the male phenotype, coupled with the stabilization of the androgen receptor in these cells, may serve as a trigger for the development of secondary sexual characteristics.

It is of special interest to us that a sequence highly homologous to a portion of the non-translated region of testican mRNA appears to be induced by testosterone. Testican is a multidomain proteoglycan for which the cDNA was first identified in testicular tissue. The protein core of testican encompasses several domains encountered in several proteins involved in adhesion, migration, and cell proliferation. Amongst these are four osteonectin-like domains, separated by non-homologous sequences of similar length. Osteonectin is a molecule involved in cell-cell and cell-matrix interactions. Within the third osteonectin-like sequence and slightly overlapping downstream, there is a 45 amino acid section highly homologous to a Kazal-domain found in rat agrin, a component of the basal lamina. Downstream of the fourth osteonectin-like domain, the testican cDNA encodes a 46 amino acid sequence with homology to a cystein rich type-I thyroglobulin repeat region including a Cys-Trp-Cys-Val sequence found as a single copy in epithelial glycoprotein, a cell surface antigen playing a key role in cell-cell or cell-matrix interactions [Alliel et al., 1993]. These data indicate that this proteoglycan may play a significant role in the morphogenesis of the male urogenital tract during development and that alterations in its biosynthesis may be involved in sexual differentiation.

A second sequence apparently upregulated by testosterone is L-plastin, an actin binding protein known to be expressed in leukocytes and malignant cells of solid tissues [Lin et al., 1993], where it appears to cause reduction in growth rate and possibly cell death [Park et al., 1994]. This supports the hypothesis that in the foreskin fibroblasts a reduction of proliferation precedes differentiation, and that organogenesis may also involve the death of specific cells. This is consistent with the observation that a sequence homologous to ubiquitin appears to be upregulated or at least no longer repressed in cells cultured in the absence of testosterone. Ubiquitin plays a central role in proteolysis, targeting proteins destined to be proteolysed [Ciechanover et al., 1994; Rechsteiner, 1991; Driscoll et al., 1992]. Ubiquitin is downregulated in a number of dedifferentiated cell types including human leukemic cells, suggesting that it may be repressed during differentiation and upregulated during dedifferentiation [Shimbara et al., 1993].

The results presented in this study suggest strongly that the extracellular matrix plays a critical role in regulating the androgenic control of gene expression. Further experiments are planned involving co-culture of epithelial cells and fibroblasts, as well as testing whether different natural and synthetic androgens such as 5a-DHT and mibolerone have the same effect on the pattern of induced genes. Moreover, investigations in genital skin fibroblasts of patients with partial androgen insensitivity due to known androgen receptor mutations are planned to find out if the pattern of the described gene expression with and without testosterone is characteristically disturbed in these patients and if a reliable correlation can be found between the clinical picture of impaired masculinization and the gene expression pattern.

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